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Fossil melanosomes or bacteria? A wealth of findings favours melanosomes

Melanin fossilises relatively readily, bacteria rarely, hence the need for clarification in the debate over the identity of microbodies in fossil animal specimens

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The discovery of fossil melanosomes has resulted in a wealth of research over the last 7 years, notably the reconstruction of colour in dinosaurs and fossil mammals. In spite of these discoveries some authors persist in arguing that the observed microbodies could represent preserved bacteria. They contend that bacteria fossilise easily and everywhere, which means that one can never be certain that a microbody is a melanosome without an extraordinary burden of evidence. However, this critique mischaracterises the morphological and structural evidence for interpreting microbodies as fossil melanosomes, and hence the basis for using them in reconstructing prehistoric colours. The claims for bacterial omnipresence in the fossil record are themselves not supported, thus tipping the scales strongly towards melanosomes in the bacteria-versus-melanosome controversy.

Keywords: prokaryote, melanin, taphonomy, false dichotomy

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Introduction

Tiny microbodies associated with integument, hair and feathers were first described from exceptionally preserved fossils in 1983 [1]. Their remarkable similarity to bacteria led to the suggestion that decay bacteria outlined these structures perfectly, preserving individual hairs and barbules as a case of auto-lithified bacteria pseudomorphing tissues in situ [1]. This interpretation highlighted the importance of bacterial activity in exceptional fossil preservation [2-4]. The role of bacteria in decay and preservation became clearer during the 1990s as studies showed how bacterial activity liberates reactive ions that precipitate on the soft tissue residues, replicating them in minerals such as apatite and pyrite [5,6]. Individual muscle fibers [7] or digestive systems [8] can be preserved through phosphatisation, while the delicate limbs and antennae of trilobites, and even ostracods with limbs and their brooded eggs [9], for example, can be preserved as a result of pyritization driven by bacterial sulphate reduction [10,11].

The role of bacteria in soft tissue preservation cannot be overstated. However, doubt about the paradigm of autolithified bacteria began to emerge before the decade was out. A well preserved 'caprimulgiform' bird was described by Gerald Mayr in 1998 [12] with clear colour patterns in its tail that closely resembled modern forms. Mayr wondered how colour patterns could survive when bacteria replace the feather. Although he did not make the link between the colour patterns and melanosomes, he strongly favoured the idea that

pigments must be preserved by some means [12]. Around the same time, the first feathered dinosaurs were described, and clear colour patterns were identified in the striped tail feathers in *Caudipteryx* [13]. In 2008 it was shown that colour patterns are preserved in feathers because the supposed bacteria represent colour-generating melanosomes [14]. This discovery has been substantiated by numerous investigations describing melanosomes in a range of taxa, ranging from the Carboniferous to the Miocene, and including detailed structural, morphological and chemical evidence [14-28].

Most recently, a group of authors have reverted to the bacterial interpretation, arguing that published studies have failed to reject the null hypothesis that the preserved microbodies represent fossil bacteria [29-31]. They assert that because bacteria are: 1) everywhere, 2) fossilise routinely and 3) are of all shapes and arrangements that overlap with melanosome morphologies, that they therefore cannot be distinguished by morphology alone. For these reasons, they argue that an extraordinary burden of proof is necessary to identify fossil melanosomes, and without this the bacterial interpretation is equally parsimonious. In their opinion geochemical evidence is required in support of any claim of fossil melanin.

Here I will show that these doubters have mischaracterised the arguments presented in published reports of fossil melanin. I will demonstrate that their hypothesis (here styled the 'microbial fossils are ubiquitous', or MFU hypothesis), is flawed and inconsistent with the evidence. And I will present a foundation for a logical basis that can be used to determine the identity of melanosomes in fossils in future.

Hierarchical evidence for fossil melanin and melanosomes

Microbodies found in melanin-bearing tissues vary from ~0.2 μm diameter granules in cephalopod ink to melanosomes that are represented as small oblong to cylindrical objects typically around 0.4 μm to 2 μm in length. The shape of melanosomes in fossil hair and feathers corresponds remarkably well with the composition of melanin. Reddish brown phaeomelanin is contained in phaeomelanosomes that are shaped like meatballs, whereas black eumelanin is contained in eumelanosomes that are shaped like sausages. Furthermore, birds can generate iridescent nanostructures using mainly eumelanosomes. This involves self assembling processes following the death of barbule cells through depletion attraction forces [32]. Due to the necessity of these melanosomes to form distinct arrangements, it has been shown that density and shape play a role in forming the structure [19]. Iridescent melanosomes are longer than black eumelanosomes and, in some cases, are flattened or hollow. Grey coloured feathers exhibit an association of melanosome morphologies distinct from iridescent, black and brown feathers. Using Canonical Discriminant Analyses with data from hundreds of modern feathers, it is possible to discriminate between these four feather colours based on melanosome shape [15,19,20]. Penguins exhibit a very distinct melanosome morphology in their black feathers, which is also statistically different [33].

In feathers, cylindrical melanosomes are generally aligned along the axis of the barbs and the barbules. Such alignment has been documented in several fossil feathers [14,15,19,26] and was used as an argument favouring their interpretation as melanosomes in fossil specimens, because bacteria do not align

themselves precisely to the feather morphology. Colour patterns are also observed in fossils. In a Cretaceous feather with transverse black and white bands, aligned melanosomes were only observed in the black bands, while the white bands preserve little but the rock matrix [14]. Another type of colour pattern is a gradient such that the base of the feather is lightly pigmented relative to the more distal part. In modern birds, this is accomplished by incorporating less melanin into the lighter parts of the feather. Such a concentration gradient was also documented in a fossil feather that shows a clear colour transition [17].

In other taxa, melanosome morphologies are less variable. In amphibians, for example, melanosomes are usually stout, ovoid structures much larger than phaeomelanosomes [16]. In vertebrate eyes, the melanosomes of the retinal pigmented epithelium (RPE) form a basal layer of ovoid melanosomes with elongate, cylindrical melanosomes in the apical part where the epithelium envelopes the photo-sensory rods and cones. Such morphological zonation has been observed in the eyes of fossil fish [21,24,34] and birds [14]. Ovoid melanosomes in vertebrate eyes were thought to be mainly eumelanin [35], but recent work using TOF SIMS suggests a mixed eu- and phaeomelanin composition within vertebrate RPE [16]. To sum up: distinct tissues and taxa host distinct melanosome assemblages.

Comparisons of melanosome impressions in fossil feathers with the melanosomes that produce them [15,33], suggest that melanosomes may shrink about 10-20% during diagenesis, and a similar reduction has been generated by experimental maturation of extant melanosomes [36]. Fortunately this shrinkage has a negligible effect [15] on colour reconstructions, as the melanosomes appear to shrink isometrically so that the most important variable in the canonical discriminant analysis, the aspect ratio, remains the same. The dimensions of melanosome impressions in the rock matrix are arguably more reliable indicators of original size as they formed earlier, and shrunken melanosomes could be reconstructed with our available knowledge [25]. Neither experiments nor fossil specimens suggest that the shape of ovoid phaeomelanosomes can be transformed to that of cylindrical eumelanosomes or vice versa. Thus colour reconstructions based on melanosome shape are still reliable.

Chemical studies of fossil melanosomes have revealed signatures diagnostic of melanin in a diversity of fossil taxa [16]: mammalian hair, feathers, frog and tadpole skin and eyes, cephalopod ink sacs and a basal cyclostome. These ranged in age from Carboniferous to Miocene and show that fossil melanosomes are similar in shape to those in their extant counterparts, and that distinct chemistries correlate with shape [16].

Contrary to postulations by the proponents of the MFU hypothesis [31], the identification of melanosomes in fossils does not rely on superficial resemblances between fossil and recent melanosome microbodies. Their identity as fossil melanosomes is confirmed by a hierarchy of evidence: The microbodies are localized in melanin bearing tissues, they show clear colour patterns, exhibit a limited morphological range, and correspond to specific taxon and tissue morphologies (in fossil feathers, for example, eumelanosomes are aligned along the axis of the barbs and barbules). Each of these observations in themselves refutes the bacterial hypothesis, and in combination they robustly

confirm that the microbodies are melanosomes. Furthermore, chemical analyses of fossil melanosomes and cephalopod ink granules recover signatures consistent with melanin [16,21-24,37-39]. Thus, the argument that these structures could be bacteria, and that chemical analysis is necessary to determine their identity in every case [21,29,31], is insufficiently informed, unless, of course, bacteria could be shown to have a high preservation potential similar to that of melanin, and would, therefore, easily be confused with melanosomes (the MFU hypothesis).

Bacteria are everywhere, and come in all sorts of sizes and shapes

Bacteria are diverse prokaryotes present in every environment, from deep in the Earth's crust to man-made spacecraft. The bacterial cell is separated from the environment by a combination of lipid-based membrane(s) and peptidoglycan cell wall (Gram positive bacteria have a lipid plasma membrane surrounded by a thick peiptidoglycan wall; Gram negative bacteria have two lipid membranes sandwiching a thin peptidoglycan wall). Enclosed by these structures is a cytoplasm with DNA, proteins and, sometimes, nutrient storage granules and protein-bound organelles (carboxysomes). Bacteria were one of the first organisms to colonize the earth, and they are adapted to all sorts of conditions and life modes. As such, bacteria are everywhere and, due to their fast reproduction rate, they can colonize any sterile surface very rapidly.

Bacteria range in dimension from 0.1 μm to millimeters [31]. They can be shaped like cylinders (bacciliforms) or spheroids (coccoids) and thus resemble melanosomes. But bacteria also take on many other shapes, including spirally coiled forms, gracile filaments, flagellated and stalked bodies. Decay experiments with feathers have shown that the naturally occurring bacteria that degrade them are similarly diverse [40], including spirals, baccilliforms and coccoids in aggregates on different parts of the feather.

If the microbodies on fossil feathers are equally likely to be bacteria, it is surprising that their morphological range does not exceed that of melanosomes. Figure 1 is a plot of melanosomes from extant and fossil samples [18] together with bacteria from the surface of an extant and partially decayed feather [29]. Supporters of the MFU have argued that these bacteria are "*similar in size, shape, distribution and location to previously published work on fossil melanosomes in side by side comparisons*" [29]. However, as Figure 1 shows, the bacteria imaged do not overlap with any recorded extant or fossil melanosome. If microbodies in fossil feathers and other melanin-bearing integument were fossilized bacteria, they should display diversity and not a uniform morphology that resembles melanosomes. Another argument put forward in support of a bacterial identity is the observation that bacteria align themselves during reproduction. But when melanosomes are aligned, they lie parallel to original barbs and barbules [14,15,26], while no study has shown that bacteria conform in orientation precisely to their substrate (magnetotacit bacteria orient themselves, but not to fossil feathers). A more pressing question is whether bacteria are commonly fossilized and might be preserved in a manner similar to melanosomes, or even *among* them.

Bacteria are everywhere, but not much in the fossil record

Fossilized bacteria and other prokaryotes provide some of the earliest evidence of life on Earth, as stromatolites and as permineralized structures in cherts, e.g [41,42]. Bacterial activity is also occasionally evident in the cemented laminae in stromatolites and thrombolites, in carbonate rocks and siliciclastic systems [43]. Due to the unstable nature of bacteria, which lyse or disintegrate within minutes after death, very rapid fossilisation is necessary. This is accomplished by early precipitation of minerals that encapsulate the microbes, such as chert [42,43], and occasionally carbonates [44] or other media such as amber [45].

The MFU hypothesis argues that bacteria fossilise easily and everywhere [31], but a survey of the geobiology literature does not support this contention. Bacteria and other prokaryotes are present in the fossil record and provide important insights into Precambrian biodiversity, but as shown by Andrew Knoll and Stjepko Golubic's [46] elegant study of a Precambrian algal stromatolite from Bitter Springs, Australia, the preservation of microbes is confined to certain conditions, which therefore results in varying degrees of degradation. In this case preservation of the microbes is due to very early precipitation of chert, which was facilitated by intermittent evaporitic conditions in intertidal stromatolites. Even within a stromatolite, certain communities were not preserved because of fluctuating preservation associated with changes in environmental conditions. This study emphasized the need for extreme conditions to facilitate early silicate precipitation of microorganisms. It is clear that these exceptional preservations provide a very biased view of the biodiversity of Precambrian microbiomes, even within a single stromatolite community [46].

An example cited in support of the MFU hypothesis [31] is the occurrence of a 1 million year old manganese stromatolite from a cave deposit in Spain [44], which preserves an extraordinary diversity of bacterial microbodies representing a variety of morphologies beyond what is observed among melanosomes. But the authors of that study acknowledged that it is a very special situation that is rarely encountered in other manganese stromatolites because of the effects of diagenesis, which normally obliterate the fine bacterial bodies [44]. Again, here the microbiome preserved exhibits a higher diversity of morphologies than observed in fossil melanosome studies. Also, Quarternary manganese stromatolites from cave deposits are not analogous to Mesozoic and Cenozoic Lagerstättens, where fossil feathers and vertebrate soft tissues are generally encountered.

Prokaryote microfossils also occur in anoxic siliciclastic settings as organically preserved microfossils, e.g. [47,48]. These are therefore preserved in the settings where vertebrate soft tissues are encountered. This occurs in forms with tough cell walls, such as cyanobacteria. However bacilliform and coccoid bacteria that superficially resemble melanosomes do not have such robust cell walls. These microfossils cannot be conflated with melanosomes.

Bacterial morphologies have occasionally been found in exceptionally preserved animal fossils. They occur in association with a Jurassic horseshoe crab, for example, in which muscle fibres are replicated in calcium phosphate [49]. These mineralized bacteria include spirally shaped trichomes and ovoid coccoids. However, even where early phosphatisation preserves muscle tissue,

associated microbes are usually not found [50]. The preservation of bacteria in a horseshoe crab [49] is more exceptional than the mineralization of the muscle fibres with which they are associated.

While fossilized bacteria occur, they are not commonly preserved in normal sedimentary environments. Bacilliforms and coccoids that resemble melanosomes are preserved when they are encapsulated immediately while still active. If the MFU hypothesis is valid, bacteria should be preserved on bedding planes in association with fossil feathers and other melanin bearing structures - on top of and next to the fossil. Unlike bacteria, melanosomes are resistant structures and can survive bacterial degradation and thermal maturation as observed in experiments [16]. Melanosomes are sometimes lost by oxidation in fossils, but they leave impressions in post-sedimentary diagenetic cements, while bacteria have long since lysed to leave such impressions.

Thus the MFU hypothesis is not supported by the geobiology literature, which shows that bacteria are preserved under exceptional conditions associated with early encapsulation, in contrast to melanosomes, which are preserved organically in fine-grained anoxic sediments. Fossil bacterial communities document a much greater diversity of morphologies than melanosome assemblages.

What is the necessary burden of proof for identifying fossilized melanosomes?

Although the melanosome-versus-bacteria controversy is demonstrably a false dichotomy, the question about how we can detect melanosomes in fossils and which basic criteria should be used to identify them is an important one. The MFU hypothesis argues that the microbodies in question are equally likely to be bacteria, and that the issue can only be resolved by chemical analyses [21,31]. Such a stance sounds appealing, why wouldn't we want to be confident in our assessment? However, the argument is presented as if fossil melanosomes characterised without chemical analysis could just as well be bacteria, while ignoring the topological and structural evidence that points to melanosomes. Melanosomes are identified in living animals in histological studies without confirmation of their chemical nature. Melanosomes are a canonical screening pigment in the retinal pigmented epithelium of vertebrates, for example, and researchers diagnose them based on their localisation in the RPE and their distinct morphology.

Chemical analyses have confirmed the presence of melanin residues in a range of fossil taxa and tissues, straddling the bilaterian tree of life (Vertebrates and Cephalopods) [16], and have showed that structures interpreted as bacteria by some [51-53] and melanosomes by others do indeed contain melanin. The material investigated in a recent study [16] included fossil feathers, frog integument and bat hair from Messel. These were the taxa that originally was interpreted as preserved through autolithified bacteria [1].

Multiple lines of evidence support the identity as melanosomes and thus only a subset of the available criteria should be necessary to present a case for fossil microbodies being melanosomes. Melanosomes in birds are well characterised in both extant forms [15,18-20] and in fossils from a series of localities. If a researcher is working on a fossil bird feather from the Eocene

Messel locality [54], do they need to perform a chemical analysis to show that the microbodies are indeed melanosomes if they conform in shape, size and arrangement to melanosomes? Two separate feathers from Messel have already been shown to contain melanin chemically [16], as have frog tissues and mammal hair. The topological and morphological observations that show that these structures conform to melanosomes are an adequate basis to argue for their identity as such. The same logic can be applied to feathers from other fossil localities. The feathers of the Jehol biota also preserve microbodies localised to melanin bearing tissues, and show the original alignment of melanosomes within feathers [19], as well as colour patterns and morphologies consistent with those in modern birds. While chemical taphonomical studies of melanin preservation from the Jehol biota is limited to a study of the older fossil, *Anchiornis* [22], future studies of melanosomes in a Jehol fossil should simply fulfil a subset of criteria available to identify melanosomes as the evidence available in Jehol is not different from evidence available from the Messel deposit, except for a deeper burial history and more meteoric weathering, which do necessitate some considerations of taphonomy, but not necessarily chemical analyses.

When do we need to perform chemical analyses? Obviously, we need to understand the taphonomy and diagenesis of melanin, which has been tackled in a number of ways [16,38,39]. Data from different sites will continue to be compiled in order to provide a better understanding of how melanin preserves and is altered through time. Research is in progress to allow eumelanin to be distinguished chemically from phaeomelanin in fossils [16]. Chemical analyses might be advisable for identification if a structure is contentious: e.g., putative eyes in a presumed basal chordate, or a case in which melanosome microbodies are merged together to form a solid mass. But for reconstructing colour patterns in, say, birds and mammals, chemical analyses are not necessary if the melanosome microbodies conform to topological and morphological observations expected for melanosomes of the taxa in question. Only if the microbodies do not resemble melanosomes, or are present outside of a conventionally accepted melanin-bearing tissue, are chemical analyses potentially crucial to confirming their identity.

One of the often cited [55] foundations for the MFU refers to objects that were initially identified as bacteria, but have since been shown unequivocally to be fossil melanosomes [1]. Almost all fossil bacterial body fossils are identified based on morphological evidence alone. Given that melanosomes have a better fossilisation potential than bacciliforms and coccoids, perhaps the onus is not on melanosome researchers to present a stronger case, but indeed the opposite. While this sounds, again, intuitive and compelling, bacteria and other prokaryotes can be identified with confidence, and they can be distinguished from melanosomes by their morphology and topology along with taphonomic and sedimentologic considerations.

Conclusions

Previous studies on fossil melanosomes [21,29,31] have been criticised on the grounds that the claimed melanosomes are equally likely to be fossilised bacteria. Here I have argued that such criticisms are largely unfounded, and are doing a disservice to a burgeoning literature on fossil melanosomes [14,17-20,25-28] and their utility in reconstructing colour. The critiques lack a holistic

representation of all the topological, morphological and structural arguments that favour the melanosome interpretation over the bacteria hypothesis [1], which was disproved at the outset in the papers that established the field [14,26]. The argument that bacteria are everywhere, and fossilise easily (the MFU hypothesis), is not supported by evidence from experiments or fossils, which show instead that the preservation of bacteria requires more exceptional circumstances than the fossilisation of melanin. Bacteria furthermore show a much more diverse range of morphologies and sizes than melanosomes. The argument that bacteria are equally likely to be preserved as melanosomes is based on a false dichotomy and an insufficiently informed interpretation of the processes involved in the fossilization of both.

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Figures

Figure 1. A representation of extant and fossil melanosomes compared with extant bacteria from a recent study by Moyer et al. [29] in support for the MFU hypothesis, claiming that these bacteria, pictured, are identical in size and shape to melanosomes. **Left** - Fossil and extant integumental melanosomes from 274 taxa including 168 birds, 36 turtles and squamates, 46 mammalians and 18 fossil taxa (Dinosaurs, birds, pterosaurs, squamates, turtles) plotted against extant bacteria on a degrading feather. The melanosome measurements are average values. **Right** – 4866 measurements from individual integumental melanosomes in mammalian, squamate, turtles and birds compared with the same bacteria. Bacterial measurements are performed on figure 2A from ref. [29] using imageJ, with the imaged scale bar for measurement calibration.